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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003904541 for a patent by THE COUNCIL OF THE QUEENSLAND INSTITUTE OF MEDICAL RESEARCH as filed on 22 August 2003.

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J. Billingsley

JULIE BILLINGSLEY
<u>TEAM LEADER EXAMINATION</u>
<u>SUPPORT AND SALES</u>

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PROVISIONAL SPECIFICATION

Invention Title: "G-CSF DERIVATIVE FOR INDUCING IMMUNOLOGICAL TOLERANCE"

The invention is described in the following statement:

TITLE

"G-CSF DERIVATIVE FOR INDUCING IMMUNOLOGICAL TOLERANCE"

FIELD OF THE INVENTION

THIS INVENTION relates to a method, composition and use thereof for inducing tolerance, including transplantation tolerance in a recipient and self tolerance in a patient. Tolerance may be induced by administration of a G-CSF derivative, in particular peg-G-CSF, to a donor or patient. Transplantation tolerance may reduce or prevent graft versus host disease or graft rejection and self tolerance may reduce or prevent an autoimmune disease.

BACKGROUND OF THE INVENTION

Allogeneic Stem Cell Transplantation (SCT) is currently indicated in treatment of a number of malignant and non-malignant diseases. However, use of allogenic SCT is limited by serious complications, the most common being graft versus host disease (GVHD). GVHD results in multi-organ damage and immune deficiency significantly impairing overall transplant survival. Use of (granulocyte-colony stimulating factor) G-CSF mobilized stem cell grafts has improved rates of immune and hematopoetic reconstitution, reduced transplant related mortality, and improved leukemia eradication after SCT (Bensinger *et al*, 2001). The mechanism by which G-CSF alters T cell function and reduces GVHD remains controversial. G-CSF has been shown to induce Th2 differentiation in donor T cells prior to SCT and this been suggested to be a major protective mechanism from GVHD in

this setting (Pan *et al*, 1995).

Despite an approximate 10-fold increase in the T cell content of G-CSF mobilised leukapheresis products compared to unstimulated bone marrow harvests (Pan *et al*, 1995; Tayebi *et al*, 2001), there is no increase in the incidence of acute GVHD (Bensinger *et al*, 2001; Ringden *et al*, 2002). Recent data suggests that CD4+ T cells exposed to G-CSF *in vivo* acquire the properties of T regulatory (Tr) cells following T cell receptor triggering *in vitro* (Rutella *et al*, 2002), although effects *in vivo* have not been explored.

Attachment of a polyethylene glycol (PEG) molecule to a protein ("pegylation") prolongs the plasma half-life of the conjugated agent (Abuchowski *et al*, 1977; Bailon *et al*, 1998), thus reducing frequency of administration of the agent. Peg-G-CSF (also known as peg-filgrastim and peg-Neupogen) has a significantly reduced rate of renal clearance and thus a longer plasma half-life than standard G-CSF (Molineux *et al*, 2003). US Patent Application 09/921114 describes treating neutropenia with peg-G-CSF.

SUMMARY OF THE INVENTION

The present inventors unexpectedly found that treating a donor with peg-G-CSF is superior to standard G-CSF for inducing tolerance, for example the prevention or reduction of GVHD. Accordingly, peg-G-CSF does not merely increase G-CSF half-life, but surprisingly also enhances G-CSF biological activity. It will be appreciated that a preferred form of the invention relates to use of any suitable agent capable of

binding and activating a G-CSF receptor, for example G-CSF and a G-CSF mimetic, including a respective fragment, homolog or variant thereof conjugated to PEG. It will also be appreciated that aspects of the invention relate to transplantation tolerance and self-tolerance.

In a first aspect, the invention provides a method for inducing transplantation tolerance, including the step of treating a donor cell with a G-CSF derivative before transplantation of the donor cell to a recipient.

Transplantation tolerance may include prevention or reduction of graft versus host disease.

The G-CSF derivative is preferably peg-G-CSF.

The G-CSF derivative may comprise recombinant G-CSF.

The G-CSF derivative preferably comprises human G-CSF.

The G-CSF derivative may comprise G-CSF or a G-CSF mimetic and respective fragment, homolog or variant thereof.

The G-CSF derivative comprising a G-CSF mimetic is preferably peg-ProGP-1.

The administered G-CSF derivative preferably comprises an amino acid sequence that is the same as or similar to that of the donor.

Suitably, the donor cell is an immune cell.

Preferably, the immune cell is a T cell and/or a granulocyte-monocyte.

Preferably, the T cell is stimulated to produce IL-10.

Preferably, the granulocyte-monocyte is characterized by a

CD11c negative phenotype.

More preferably, the granulocyte-monocyte is further characterized by a $CD11b^{hi}Gr\text{-}1^{dim}$ phenotype.

Preferably, the granulocyte-monocyte characterized by a $CD11b^{hi}Gr\text{-}1^{dim}$ phenotype increases in number and/or frequency in the donor by treating said donor with the G-CSF derivative.

The donor cell may be treated with the G-CSF derivative *in vivo* and/or *in vitro*.

The donor cell is preferably treated with the G-CSF derivative *in vivo* by administering to the donor said G-CSF derivative.

Preferably, the G-CSF derivative is administered to the donor *in vivo* as a single dose.

Preferably, the single dose is administered in a range from 3-60 µg/animal.

The donor cell may be isolated from the donor after *in vivo* administration of the G-CSF derivative and before transplantation of the donor cell to the recipient.

The donor cell may be purified as a homogeneous cell population after *in vivo* administration of the G-CSF derivative and before transplantation of the donor cell to the recipient.

The method may further include the step of propagating the isolated or purified donor cell *in vitro* before transplantation of the donor cell to the recipient.

Preferably, the donor is a mammal.

More preferably, the mammal is a human.

Preferably, other cells are transplanted from the donor to the recipient in addition to the donor cell treated with the G-CSF derivative.

More preferably, the other cells are transplanted simultaneously with the donor cell treated with the G-CSF derivative.

The other cells may include a single cell suspension, unseparated cells, tissue or organ.

The other cells may be purified as a homogeneous cell population before transplantation.

The other cells may be propagated *in vitro* prior to transplantation of the other cells to the recipient.

The other cells may be a population of stem cells.

Preferably, the stem cells are hematopoetic stem cells.

In one form, the method includes the steps of:

(1) isolating the donor cell; and

(2) activating the isolated donor cell *in vitro* with the G-CSF derivative before transplantation of the isolated donor cells to the recipient.

Preferably, the isolated donor cell is a T cell.

In a second aspect, the invention provides a method for stimulating a donor cell to produce IL-10 to thereby induce transplantation tolerance including the step of treating the donor cell with a G-CSF derivative before transplantation of the donor cell to a recipient.

Preferably, the donor cell is a T cell.

The G-CSF derivative may comprise recombinant G-CSF.

The G-CSF derivative is preferably peg-G-CSF.

The peg-G-CSF preferably comprises human G-CSF.

The administered G-CSF derivative preferably comprises an amino acid sequence that is the same as or similar to that of the donor.

Preferably, the donor is a mammal.

More preferably, the mammal is a human.

Preferably, the G-CSF derivative is administered *in vivo* to the donor before transplantation of the donor cell to the recipient.

In a third aspect, the invention provides a method for inducing self-tolerance in a patient including the step of treating a cell of the patient with a G-CSF derivative.

Suitably, inducing self-tolerance in the patient treats or reduces an autoimmune disorder of the patient.

The autoimmune disorder may include rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and inflammatory bowel disease.

Suitably, the cell of the patient is an immune cell.

Preferably, the immune cell is a T cell and/or a granulocyte-monocyte.

The cell of the patient is preferably treated with the G-CSF derivative by *in vivo* administration of said G-CSF derivative to the patient.

The cell of the patient may be propagated *in vitro*, treated with the G-CSF derivative after propagation and then administered to the patient.

Preferably, the cell of the patient is stimulated to produce IL-10.

The G-CSF preferably comprises human G-CSF.

The G-CSF derivative is preferably peg-G-CSF.

In a fourth aspect, the invention provides a method for inducing transplantation tolerance including the step of treating a donor cell with a G-CSF derivative before transplantation of a donor cell to a recipient, wherein transplantation tolerance is enhanced when compared with treating a donor cell with G-CSF.

Enhanced transplantation tolerance includes an increase in survival of the recipient.

Transplantation tolerance may include prevention or reduction of graft versus host disease.

In a fifth aspect, the invention provides a composition comprising a cell treated according to the method of any one of the preceding aspects.

Preferably, the cell is an immune cell.

More preferably, the immune cells is a T cell and/or a granulocyte-monocyte.

Preferably, the granulocyte-monocyte is characterized by a CD11c negative phenotype.

More preferably, the granulocyte-monocyte is further

characterized by a $CD11b^{hi}Gr\text{-}1^{dim}$ phenotype.

In a sixth aspect, the invention provides use of the composition of the fifth aspect to induce tolerance in a patient.

Tolerance includes transplantation tolerance and self-tolerance.

Transplantation tolerance may prevent or reduce graft versus host disease.

Self tolerance may prevent or reduce an autoimmune disorder.

The autoimmune disorder may include rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and inflammatory bowel disease.

In a seventh aspect, the invention provides use of a pharmaceutical composition comprising a G-CSF derivative for inducing transplantation tolerance by administering said pharmaceutical composition to a donor before transplantation of a donor cell to a recipient.

Preferably, the G-CSF derivative is peg-G-CSF.

Preferably, transplantation tolerance is enhanced when compared with administering a pharmaceutical composition comprising G-CSF.

Enhanced transplantation tolerance includes an increase in survival of the recipient.

In an eighth aspect, the invention provides use of a pharmaceutical composition comprising a G-CSF derivative for inducing self-tolerance by administering said pharmaceutical composition to a patient.

Preferably, the G-CSF derivative is peg-G-CSF.

Preferably, self-tolerance is enhanced when compared with administering a pharmaceutical composition comprising G-CSF.

Enhanced transplantation tolerance includes an increase in survival of the patient.

Throughout this specification unless the context requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of the stated integers or group of integers or steps but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES

In order that the invention may be readily understood and put into practical effect, preferred embodiments will now be described by way of example with reference to the accompanying figures.

FIG. 1A: Survival by Kaplan-Meier analysis. Donor B6 mice were treated for 6 days with human G-CSF (0.2ug/animal, 2µg/animal or 10µg/animal) or control diluent. T cell dose was equilibrated across all groups (3 x10^6 T cells/recipient). Splenocytes were harvested on day 7 and transplanted into lethally irradiated (1100 cGy) B6D2F1 recipient mice (control syngeneic recipients n=6; control allogeneic n=6; G-CSF 0.2µg/day n=12; G-CSF 2.0µg/day n=12; G-CSF 10µg/day n=6). *P*=0.03, 0.2µg G-CSF versus 2µg G-CSF; *P*=0.004, 0.2µg G-CSF versus 10µg G-CSF. Combined results from two identical experiments shown.

FIG. 1B: Survival by Kaplan-Meier analysis. Donor B6 mice were treated with murine G-CSF (0.2μg/animal, 0.5μg/animal or 2μg/animal for 6 days) or control diluent and transplanted as above. B6D2F1 recipient mice (control syngeneic recipients n=6; control allogeneic n=6; murine G-CSF 0.2μg/day n=6; murine G-CSF 0.5μg/day n=6; murine G-CSF 2μg/day n=12). Survival P=0.003, 0.2μg murine G-CSF versus 2μg murine G-CSF. Combined results from 2 identical experiments shown.

FIG. 2A: Survival by Kaplan-Meier analysis. Donor B6 mice received either control diluent, 2μg standard human G-CSF daily for 6 days, 3μg peg-G-CSF or 12μg peg-G-CSF as a single injection on day –6. Lethally irradiated B6D2F1 recipient mice were transplanted as in FIG. 1 (control syngeneic recipients n=6; control allogeneic n=6; peg-G-CSF 3μg n=6; peg-G-CSF 12μg n=6; human standard G-CSF 2μg/day n=18. P=0.82, 3μg peg-G-CSF versus 12μg peg-G-CSF, P=0.0001, 2μg G-CSF (for 6 days) versus 12μg peg-G-CSF (single dose).

FIG. 2B: GVHD clinical scores were determined as a measure of GVHD severity in surviving animals as described herein. *P<0.05 for 2μg human G-CSF (6 days) versus 12μg peg-G-CSF (single dose). Combined results from 2 identical experiments shown.

FIG. 3A: Splenocyte expansion following donor pre-treatment with standard or pegylated G-CSF shown as relative proportions of each cell lineage. Donor B6 mice (n=4 per group) received either control diluent, 2μg human G-CSF/day for 6 days or single injection of 12μg peg-G-CSF day –6

and splenocytes were harvested on day 7.

FIG. 3B: Splenocyte expansion following donor pre-treatment with standard or pegylated G-CSF shown as absolute numbers of each cell lineage. *$P<0.05$* control versus peg-G-CSF, +$P<0.05$ peg-G-CSF versus control and G-CSF. Data presented as mean ± SD.

FIG. 4A: Donor treatment with peg-G-CSF impairs T cell function and induces regulatory T cell activity. C57BL/6 T cells from control, G-CSF 2μg/day for 6 days or peg-G-CSF 12μg single dose day -6 were stimulated at ratios as shown with irradiated B6D2F1 peritoneal macrophages. Proliferation was measured at 72 hours via standard [^{3}H] Thymidine incorporation assay. $P<0.05$ control versus G-CSF and $P<0.05$ control versus peg-G-CSF. IFN-γ and IL-2 production were determined in culture supernatants by ELISA.

FIG. 4B: Non-cytokine exposed C57BL/6 T cells were stimulated with irradiated B6D2F1 macrophages. Additional T cells from wild-type C57BL/6 donors pre-treated with control diluent or peg-G-CSF 12μg day –6, or from IL-10$^{-/-}$ donors pre-treated with peg-G-CSF 12μg day –6, were added at doses as shown. Proliferation was measured at 72 hours via standard [^{3}H] Thymidine incorporation assay. *$P<0.05$ control versus wild-type peg-G-CSF.

FIG. 4C: Whole spleen from control, G-CSF, or peg-G-CSF pre-treated donors as above was stimulated with LPS and CPG, and IL-10 measured in supernatants at 48 hours by ELISA. $P=0.0002$ control versus G-

CSF; P=0.001 control versus peg-G-CSF. Data (FIGS. 11A-11C) presented as mean ± SD of triplicate wells and represents one of two identical experiments.

FIG. 5A: Survival by Kaplan-Meier analysis. P<0.001 for wild-type TCD spleen + wild-type T cells versus wild-type TCD spleen + IL10$^{-/-}$ T cells; P<0.0001 IL10$^{-/-}$ TCD spleen + wild-type T cells versus IL10$^{-/-}$ spleen + IL10$^{-/-}$ T cells. *FIG.* 5A shows protection from GVHD afforded by peg-G-CSF is dependant on donor T cell production of IL-10. Donors were pre-treated with a single dose of 12g peg-G-CSF at day –6. T cell depleted (TCD) splenocytes from wild-type or IL-10$^{-/-}$ donors plus purified CD3^{pos} T cells from wild-type or IL-10$^{-/-}$ B6 donors were combined as indicated, and injected into lethally irradiated B6D2F1 recipients (wild-type TCD spleen only n=6, wild-type T cells plus wild-type or IL-10$^{-/-}$ spleen n=15, IL-10$^{-/-}$ T cells plus wild-type or IL-10$^{-/-}$ spleen n=13).

FIG. 5B: GVHD clinical scores determined as a measure of GVHD severity in surviving animals. **P<0.05* wild-type TCD spleen + IL10$^{-/-}$ T cells versus wild-type TCD spleen + wild-type T cells.

FIG. 6: The protective IL-10 producing donor T cell has regulatory function. Lethally irradiated B6D2F1 recipients received splenocytes from control treated wild-type B6 donors plus additional purified T cells from control or cytokine pre-treated donors, as shown (syngeneic control n=3; allogeneic control n=5; wild-type allogeneic control + wild-type control pre-treated T cells n=9; wild-type allogeneic control + wild-type G-

CSF pre-treated T cells n=10; wild-type allogeneic control + wild-type peg-G-CSF pre-treated T cells n=14; wild-type allogeneic control + IL-10-/- peg-G-CSF pre-treated T cells n=13). Survival by Kaplan-Meier analysis. P<0.0001 wild-type allogeneic control + wild-type peg-G-CSF pre-treated T cells versus wild-type allogeneic control + wild-type control pre-treated T cells; P<0.0001 wild-type allogeneic control + wild-type peg-G-CSF pre-treated T cells versus wild-type allogeneic control + wild-type G-CSF pre-treated T cells; P<0.0001 wild-type allogeneic control + wild-type peg-G-CSF pre-treated T cells versus wild-type allogeneic control + IL-10-/- peg-G-CSF pre-treated T cells. Data combined from 2 identical experiments.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have a meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any method and material similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purpose of the present invention, the following terms are defined below.

The present invention relates to an unexpected finding that treating a donor with peg-G-CSF is markedly superior to G-CSF for the induction of transplantation tolerance and prevention of GVHD. Accordingly, peg-G-CSF does not merely increase G-CSF half-life, but surprisingly also enhances G-CSF biological activity. It will be appreciated

that PEG may be conjugated to any suitable form of G-CSF, including for example, a G-CSF fragment, homolog, variant and mimetic, such as ProGP-1.

G-CSF, G-CSF derivatives and G-CSF mimetics

By "*protein*" is also meant "*polypeptide*", either term referring to an amino acid polymer, comprising natural and/or non-natural D- or L-amino acids as are well understood in the art. G-CSF may be referred to as both a protein or polypeptide. Protein may refer to a peptide or fragments thereof, for example a fragment of G-CSF.

"*G-CSF*" refers to G-CSF protein and fragments, homologs and variants thereof. G-CSF protein is distinct from a G-CSF derivative, for example peg-G-CSF, by not being artificially conjugated to another molecule, for example PEG as described herein. G-CSF protein may comprise naturally occurring modification such as glycosylation. G-CSF may be derived from any species, including human, mouse, rat and others. G-CSF may be recombinant or native and may comprise natural and/or non-natural D- or L-amino acids as are well understood in the art.

The protein may be isolated, for example, G-CSF or G-CSF derivative and other proteins may be removed from their natural state or be synthetically made or recombinantly expressed. A "*peptide*" is a protein having no more than fifty (50) amino acids.

In one embodiment, a "*fragment*" includes an amino acid sequence that constitutes less than 100%, but at least 20%, preferably at

least 30%, more preferably at least 80% or even more preferably at least 90% of said polypeptide.

The fragment includes a "*biologically active fragment*", which retains biological activity of a given protein or peptide. For example, a biologically active fragment of G-CSF capable of inducing tolerance in a subject may be used in accordance with the invention. The biologically active fragment constitutes at least greater than 1% of the biological activity of the entire polypeptide or peptide, preferably at least greater than 10% biological activity, more preferably at least greater than 25% biological activity and even more preferably at least greater than 50% biological activity.

As used herein, "*variant*" proteins are proteins in which one or more amino acids have been replaced by different amino acids. Protein variants of G-CSF that retain biological activity of native or wild type G-CSF may be used in accordance with the invention. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the polypeptide (conservative substitutions). Generally, the substitutions which are likely to produce the greatest changes in a polypeptide's properties are those in which (a) a hydrophilic residue (*e.g.*, Ser or Thr) is substituted for, or by, a hydrophobic residue (*e.g.* Leu, Ile, Phe or Val); (b) a cysteine or proline is substituted for, or by, any other residue; (c) a residue having an electropositive side chain (*e.g.*, Arg, His or Lys) is substituted for, or by, an

electronegative residue (*e.g.*, Glu or Asp) or (d) a residue having a bulky side chain (*e.g.*, Phe or Trp) is substituted for, or by, one having a smaller side chain (*e.g.*, Ala, Ser)or no side chain (*e.g.*, Gly).

As generally used herein, a "*homolog*" shares a definable nucleotide or amino acid sequence relationship with another nucleic acid or polypeptide as the case may be. A "*protein homologs*" share at least 80%, preferably at least 90% and more preferably at least 95% sequence identity with the amino acid sequences of polypeptides as described herein. Homologs of G-CSF may also be used in accordance with the invention. Such G-CSF homologs would preferably be characterized by biological activity about the same or greater than that of a G-CSF protein having a high or substantial biological activity.

"*Orthologs*" are included within the scope of homologs. Orthologs are functionally-related proteins and their encoding nucleic acids, isolated from other organisms or species. For example, human G-CSF is an ortholog of mouse G-CSF. It will be appreciated that a protein ortholog may be administered to a donor and retain biological activity. However, it is preferred that the G-CSF administered comprises an amino acid sequence that is the same or similar to that of the donor. More preferably, the G-CSF is human G-CSF. An example of a suitable human G-CSF is described in US Patent Application 09/921,114, incorporated herein by reference.

With regard to protein variants, these can be created by mutagenising a polypeptide or by mutagenising an encoding nucleic acid,

such as by random mutagenesis or site-directed mutagenesis. Examples of nucleic acid mutagenesis methods are provided in Chapter 9 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel *et al.*, *supra* which is incorporated herein by reference.

As used herein, *"derivative"* proteins are proteins that have been altered, for example by conjugation or complexing with other chemical moieties or by post-translational modification techniques as would be understood in the art. A preferred derivative includes G-CSF conjugated to polyethylene glycol (PEG) (i.e. "pegylation"), resulting in peg-G-CSF as described herein. It will be appreciated that PEG may be conjugated to any suitable agent capable of binding and activating a G-CSF receptor, for example forms of G-CSF, including for example, a G-CSF fragment, biologically active fragment, homolog, ortholog, variant and G-CSF mimetic, such as ProGP-1. A preferred form of peg-G-CSF and methods for making peg-G-CSF are described in US Patent Application 09/921,114, incorporated herein by reference. As described in the US Patent Application, PEG may be covalently bound to amino acid residues of G-CSF, preferably human G-CSF. The amino acid residue may be any reactive one having, for example, free amino or carboxyl groups, to which a terminal reactive group of an activated polyethylene glycol may be bound. The amino acid residues having the free amino groups may include lysine residues and N-terminal amino acid residue, and those having the free carboxyl group may include aspartic acid, glutamic acid residues and C-terminal amino acid residue. A molecular

weight of PEG is not limited to any particular range; however, a suitable range includes from 500-20,000 and preferably of from 4,000-10,000. PEG may be bound to G-CSF via a terminal reactive group or a spacer. The spacer may mediate a bond between the free amino or carboxyl groups and polyethylene glycol. Peg-G-CSF may be purified from a reaction mixture using methods common in the art for purifying proteins, such as affinity purification, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel chromatography and electrophoresis. Ion-exchange chromatography is particularly effective in removing unreacted polyethylene glycol and human G-CSF.

Derivatives also comprise amino acid deletions and/or additions to polypeptides of the invention, or variants thereof. "*Additions*" of amino acids may include fusion of the protein with amino (N) and/or carboxyl (C) terminal amino acids "tags".

Other derivatives contemplated by the invention include, modification to amino acid side chains, incorporation of unnatural amino acids and/or their derivatives during peptide or polypeptide synthesis and the use of cross linkers and other methods which impose conformational constraints on the polypeptides, fragments and variants of the invention.

The term "*mimetic*" is used herein to refer to molecules that are designed to resemble particular functional regions of proteins or peptides, and includes within its scope the terms "*agonist*", "*analogue*" and "*antagonist*" as are well understood in the art.

An "*agonist*" refers to a molecule, such as a drug, enzyme activator or protein, which enhances activity of another molecule or receptor site. For example, progenipoietin-1 (ProGP-1) is a synthetic chimeric molecule that stimulates both G-CSF and Flt-3L receptors (Streeter *et al*, 2001; Fleming *et al*, 2001). Accordingly, ProGP-1 is a preferred G-CSF agonist capable of triggering a biological activity greater than G-CSF.

Cells used in relation to the invention

For the purposes of this invention, by "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state.

Cells used in relation to the invention may be isolated from a donor before and/or after treatment with the G-CSF derivative. The isolated cells may be isolated from blood using well know methods in the art. The isolated cells may form part of a tissue or organ, for example a biopsy from bone or any other tissue. Accordingly, isolated cells may comprise an isolated heterogeneous population of cells, an isolated homogeneous population of cells, cell suspension, unseparated cells and other forms of isolated cells well known in the art. It will be appreciated that use of the term "cell" includes one or more cells, for example a single cell, a population of cells and a group of cells that may form a tissue or organ.

Isolated material includes cells that have been "*enriched*" or "*purified*", meaning a population of cells comprising a higher percentage of a particular cell type when compared with other individual cell types from a same tissue or origin. A purified cell population may be homogeneous for a selected cell type. A "*homogeneous cell population*" preferably comprises a single cell type comprising at least 25% of the total isolated cell population, at least 50%, at least 75%, at least 80%, at least 90% and even greater than 98% of the total isolated cell population. For example, T cells and/or stem cells may be enriched from spleen or any other suitable tissue or organ.

Cells may be purified using any suitable method known in the art, including for example, affinity purification using a ligand, protein, antibody (either monoclonal or polyclonal) or any other suitable binding agent capable of binding to a selected cell. The binding agent may be attached to a substrate such as a matrix, bead (including a magnetic bead), solid surface or any other suitable surface. The cells may be purified using an affinity column, panning, FACS and like methods known in the art. Cells may be purified by cell deletion by binding unwanted bound cells to a binding agent and discarding the bound cells. Alternatively, or in addition, cells may be purified by positively selecting cells by binding wanted cells to a binding agent and collecting the bound cells. The bound cells may further be removed from the binding agent. Cells may be purified by separation based on size, density or other physical property, for example by density gradient, including nycodenz density gradient. Cells, for example T cells, may be

purified by teased nylon wool column purification.

"*Stem cell*" as used herein refers to a "*multipotent*" cell capable of giving rise to many different types of cells. A stem cell may be obtainable from any suitable source, including for example, spleen, blood, bone marrow, skin, nasal tissue, hair follicle and any other source. A stem cell may be an allogeneic stem cell. Stem cells may be used in allogeneic stem cell transplantation (SCT) as is known in the art. The G-CSF derivate treated donor T cells and/or granulocyte-monocyte may be transplanted with allogeneic stem cells to reduce or prevent GVHD.

By "*antigen presenting cell*" (APC) is meant a cell that displays a foreign antigen on its cell surface, typically bound to a class II glycoprotein. The foreign antigen may be recognized by a helper T cell. A granulocyte-monocyte and dendritic cell are APC.

By "*T cell*" is also meant "*T lymphocyte*", which refers to a thymus-derived lymphocyte involved with cell-mediated immune responses. T cell includes: cytotoxic T cells, regulatory T cells, helper T cells and suppressor T cells.

By "*granulocyte-monocyte*" ("GM") is meant a type of white blood cell, namely a precursor cell in the developmental pathway of becoming a monocyte. Preferably, a GM cell as used herein is characterized by a CD11c negative phenotype. More preferably, the GM cell is further characterized by a $CD11b^{hi}Gr\text{-}1^{dim}$ phenotype.

By "*dendritic cell*" (DC) is meant a type of APC that have a

function in the development of immune responses against microbial pathogens and tumors. Subpopulations of DC may be present in the thymus, spleen, Peyer's patches, lymph nodes and skin. A DC cell preferably positively expresses CD11c.

Cells used in relation to the invention, either treated and/or untreated, may be propagated *in vitro* before transplantation. Cells may be propagated using tissue culture methods that are well known in the art. Cells may be propagated on any suitable surface, including tissue culture in flasks, plates, wells, roller bottles and other known means in the art. The surface may be uncoated, glass, polymer or coated with a suitable molecule such as a matrix (eg extracellular matrix), charged particle (eg poly-l-lysine) and the like that may be selected by a skilled person. The cells may be propagated in culture media comprising actives including antibiotics, growth factors, cytokines and other actives that may increase the rate of cell division, differentiate the cells into a selected cell type and/or maintain a cell as a selected cell type.

Compositions in relation to the invention

A "*composition*" includes a "*pharmaceutical composition*", which comprises an active for delivery to a subject. The active may be a protein such as G-CSF, or fragment, homolog, variant or derivative thereof, such as peg-G-CSF, which stimulates biological activity. A preferred form of a pharmaceutical composition comprises a G-CSF derivative, more preferably peg-G-CSF. However, the composition may comprise other forms of G-CSF,

including for example a G-CSF mimetic, such as PrGP-1, and respective fragments, variants and homologs thereof conjugated to PEG. The composition may further comprise non-PEG forms of G-CSF and G-CSF mimetics, and respective fragments, variants and homologs thereof.

The composition may also comprise as an active one or more cells, for example one or more donor cells such as donor T cells that have been treated with the G-CSF derivative (eg peg-G-CSF). It will be appreciated that in addition to treatment with the G-CSF derivative, the cells of the composition may further be treated with G-CSF and/or a G-CSF mimetic such as ProGP-1. The composition may comprise a homogeneous population of cells treated in accordance with the invention. For example, the composition may comprise a homogeneous population of T cells treated in accordance with the invention. The composition may comprise a heterogeneous population of cells. The heterogeneous population of cells may be non-purified cells. The heterogeneous population of cells may comprise two or more homogenous population of cells that have been combined to thereby form the heterogeneous population. For example, a homogenous population of treated donor T cells and a homogeneous population of allogeneic stem cells. The composition may comprise a heterogeneous population of cells wherein some of the cells have been treated in accordance with the invention, for example T cells, and untreated cells, for example allogeneic stem cells.

Suitably, the pharmaceutical composition comprises a

pharmaceutically-acceptable carrier. By *"pharmaceutically-acceptable carrier, diluent or excipient"* is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including phosphate buffered solutions, sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, emulsifiers, isotonic saline, and pyrogen-free water.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Delivery of compositions of the invention

Any suitable route of administration may be used for providing an individual with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular,

intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed.

Preferably, the G-CSF derivative is administered by subcutaneous injection of the donor.

A preferred form of administration of a composition comprising cells treated in accordance with the methods of the invention is by intravenous injection. However, other routes of administration may be used as described above. The composition may be administered to a recipient at a site of solid organ transplantation during transplantation. The composition of the invention may further include any other suitable agent, for example an antibiotic, immune suppressing agent, cytokine or any other agent selected by a skilled person that may assist in preventing GVHD and improve survival and recovery of the recipient.

The cells of the composition may be propagated *in vitro* to increase cell number as described above before transplantation.

The cells of the composition may be treated *in vivo* by administration of G-CSF derivative to the donor before transplantation of donor cells to the recipient. It will be appreciated that in addition to treatment with the G-CSF derivative, the cells of the composition may further be treated with G-CSF and/or a G-CSF mimetic such as ProGP-1. A suitable route of administration may be selected by a person skilled in the art, including routes described above. Administration may be via dosage forms as described hereinafter. The cells of the composition may be treated *in vitro* by exposure

to G-CSF, G-CSF derivative or G-CSF mimetic, for example addition of G-CSF, G-CSF derivative or G-CSF mimetic to cell culture media during cell culturing. It will be appreciated that alternatively, or in addition to the above agents, other suitable growth factors, cytokines, antibiotics and agents may be added to the culture media to improve propagation and cell survival. Cells treated in accordance with the invention suitably produce IL-10. IL-10 may be measured using well known methods as herein described including by ELISA using antibodies specific for IL-10. Such antibodies may be monoclonal or polyclonal.

Compositions of the present invention suitable for administration may be presented as discrete units such as vials, capsules, sachets or tablets each containing a pre-determined amount of one or more immunogenic agent of the invention, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more immunogenic agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the agents of the invention with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

Transplantation

It will be appreciated that although the experiments described herein describe transplantation of spleen derived stem cells to a recipient, other cell types may be transplanted. For example, stem cells isolated from blood, bone marrow, skin, hair follicle or any other suitable source. The invention may also be used in relation to solid organ transplantation, such as transplantation of heart, lung, liver, kidney, skin or any other suitable organ or tissue. Methods for transplantation are well known any suitable transplantation method may be used in accordance with the invention.

Further, the invention may relate to treating autoimmune disorders wherein transplantation is omitted. Examples of autoimmune disorders include rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and inflammatory bowel disease. In one form for treating an autoimmune disorder, the G-CSF derivative may be administered to the patient, for example by injection. The administration of the G-CSF derivative may activate T cells directly or indirectly to produce IL-10.

The patient's cells, for example T cells of the patient may be propagated *in vitro* to thereby increase the number of T cells before administration thereof. This may be particularly useful in the setting of autoimmunity where there has been a breakdown in active regulatory tolerance to self.

"*Graft versus host disease*" (GVHD) also refers to "graft versus host reaction" meaning, a reaction wherein immunocompetent cells from a

donor transplant immunologically react with antigens of the recipient. GVHD typically occurs following allogeneic stem cell transplantation due to HLA disparity between donor and recipient. Donor T cells treated in accordance with the invention are particularly useful in preventing or reducing GVHD, thereby improving recovery and survival of the recipient. It will understood that the invention does not need to totally prevent GVHD or completely render an animal immunologically tolerant to be useful.

"*Tolerance*" also refers to "immunological tolerance, immuno-tolerance and non-responder tolerance" meaning a decrease in, or loss of, an ability of an animal to produce an immune response upon administration of an antigen. Theories of tolerance induction include clonal deletion and clonal anergy. In clonal deletion, the actual clone of cells is eliminated whereas in clonal anergy the cells are present, but are immunologically nonfunctional. Tolerance may also refer to a decrease in, or loss of, an ability of immuno-competent cells from a donor to produce an immune response, for example a decrease in, or loss of, GVHD. A preferred embodiment of the present invention relates to a method for inducing transplantation tolerance, however, it will be appreciated that another preferred embodiment of the invention relates to treating an autoimmune disorder of a patient wherein transplantation is omitted.

The present invention may be particularly useful with allogeneic transplantation, for example allogeneic stem cell, tissue or organ transplantation, because allogeneic transplantation typically results in GVHD.

Allogeneic transplantation refers to transplantation of a cell, organ or tissue that is donated either by a genetically matched donor such as a relative of the patient or by an unrelated (but often genetically similar) donor. Two or more individuals are considered to be allogeneic to one another when the genes at one or more loci are not identical in sequence in each organism.. It will be appreciated that the invention may also be used in relation to syngneic transplantation.

The donor and recipient of a transplant are preferably mammals, including for example humans, primates, livestock (eg cattle, sheep, pigs), race animals (eg horse, dog, camel), domesticated companion animals (eg dogs, cats) and research animals (eg mice, rats, rabbits, goats). The mammal is preferably human. Preferably, the donor and recipient are the same species, although transplantation between species, ie xenotransplantation, falls within the scope of transplantation.

The composition of cells as describe above are preferably used in transplantation to prevent or reduce GVHD. The cells treated in accordance with the invention are preferably transplanted at the same time as other cells, for example stem cells or solid tissue or organ. However, the cells treated in accordance with the invention may be transplanted before, simultaneously (eg co-administered) and/or after transplantation of other cells. A single pharmaceutical composition may comprise a plurality of cells to be simultaneously transplanted. Alternatively, a plurality of cells may be simultaneously transplanted by simultaneous administration of two or more

pharmaceutical compositions, each comprising one or more cell types.

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

EXAMPLE 1

Methods

Mice. Female C57BL/6 (B6, H-2b, Ly-5.2+), B6 PTRCA Ly-5a (H-2b, Ly-5.1+) and B6D2F1 (H-2b/d, Ly-5.2+) (Morse *et al*, 1987) mice were purchased from the Australian Research Centre (Perth, Western Australia, Australia). C57BL/6 IL-10-/- mice (B6, H-2b, Ly-5.2+) supplied by the Australian National University (Canberra, Australia). The age of mice used as BMT recipients ranged between 8 and 14 weeks. Mice were housed in sterilised microisolator cages and received acidified autoclaved water (pH 2.5) and normal chow for the first two weeks post BMT.

Cytokine treatment. Murine G-CSF (Amgen, Thousand Oaks, CA, USA), recombinant human G-CSF (Amgen, Thousand Oaks, CA, USA), pegylated recombinant human G-CSF (peg-G-CSF) (Amgen, Thousand Oaks, CA, USA) or control diluent was diluted in 1ug/ml of murine serum albumin in PBS before injection. Mice were injected subcutaneously with doses of murine or human G-CSF from days –6 to –1, or peg-G-CSF on day –6 at doses as stated.

Stem Cell Transplantation. Mice were transplanted according to a standard protocol as has been described previously (Pan *et al*, 1995; Pan *et al*, 1999),

both incorporated herein by reference. Briefly, on day –1, B6D2F1 mice received 1100cGy total body irradiation (137Cs source at 108 cGy/min), split into two doses separated by 3 hours to minimise gastrointestinal toxicity. Donor splenocytes resuspended in 0.25 ml of Leibovitz's L-15 media (Gibco BRL, Gaithersburg MD) were injected intravenously into recipients. T cell depletion (via 2 cycles of anti-CD4, anti-CD8 and anti-Thy1.2 plus rabbit complement) or T cell purification (via teased nylon wool column purification) were performed as indicated. Survival was monitored daily, and GVHD clinical score were measured weekly.

Assessment of GVHD. The degree of systemic GVHD was assessed by a scoring system which sums changes in five clinical parameters: weight loss, posture (hunching), activity, fur texture and skin integrity (maximum index = 10) (Hill *et al*, 1997; Hill *et al*, 1998; Hill *et al*, 1999). Individual mice were ear-tagged and graded weekly from 0 to 2 for each criterion without knowledge of treatment group. Animals with severe clinical GVHD (scores > 6) were sacrificed according to ethical guidelines and the day of death deemed to be the following day.

FACS analysis. Fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (mAb) CD3, CD4, CD8, CD11b, CD11c, class II, B220 and identical phycoerythrin (PE) conjugated antibodies were purchased from PharMingen (San Diego, CA, USA). Cells were first incubated with mAb 2.4G2 for 15 minutes at 4°C, then with the relevant conjugated mAb for 30 minutes at 4°C. Finally, cells were washed twice with PBS/0.2% BSA, fixed

with PBS/1% paraformaldehyde and analysed by FACScan (Becton Dickinson, San Jose, CA, USA).

Cell cultures. Culture media additives were purchased from Gibco BRL (Gaithersburg, MD, USA) and media was purchased from Sigma (St Louis, MO, USA). Cell culture was performed in 10% FCS / RPMI supplemented with, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, 0.02 mM β-mercaptoethanol, and 10 mM HEPES, pH 7.75 at 37°C in a humidified incubator supplemented with 5% CO2. For in vitro allo-antigen experiments, purified B6 T cells were cultured in round bottom 96 well plates (Falcon, Lincoln park, NJ, USA) with 105 irradiated (2000cGy) F1 peritoneal macrophages (primary MLC) and supernatants harvested at 72 hours. Cultures were then pulsed with 3H-thymidine (1 μCi per well) and proliferation was determined 16 hrs later on a 1205 Betaplate reader (Wallac, Turku, Finland). For in vitro mitogen stimulation, purified B6 T cells were cultured in flat bottomed 96 well plates, pre-coated with monoclonal CD3 and CD28 at final concentrations of 10μg/ml. Supernatants were harvested at 48 hours and cultures pulsed with 3H-thymidine (1 μCi per well). Proliferation was determined 16 hrs later on a 1205 Betaplate reader (Wallac, Turku, Finland). In secondary MLC, purified T cells were cultured in flat bottom 24 well plates (Falcon, Lincoln park, NJ, USA) with irradiated (2000cGy) splenocytes. Six days later, cells were removed and restimulated with F1 macrophages. Supernatants were removed 24hrs later and 3H-

thymidine added as above.

Cytokine ELISAS. The antibodies used in the TNFα, IFNγ, IL-10 and IL-4 assays were purchased from PharMingen (San Diego, CA, USA). All assays were performed according to the manufacturer's protocol. Briefly, samples were diluted 1:3 to 1:24 and TNFα, IFNγ, IL-10 and IL-4 proteins were captured by the specific primary monoclonal antibody (mAb), and detected by biotin-labelled secondary mAbs. The biotin-labelled assays were developed with strepavidin and substrate (Kirkegaard and Perry laboratories, Gaithersburg, MD, USA). Plates were read at 450 nm using a microplate reader (Bio-Rad Labs, Hercules, CA, USA). Recombinant cytokines (PharMingen) were used as standards for ELISA assays. Samples and standards were run in duplicate and the sensitivity of the assays was 16 to 20 pg/ml for TNFα, 0.063 U/ml for IFNγ, and 15 pg/ml for IL-10 and IL-4. Supernatants were collected after 4 hours of culture for TNFα 40 hours for IL-4, IL-10 and IFNγ analysis. Serum was stored at –70 C until analysis.

Statistical analysis. Survival curves were plotted using Kaplan-Meier estimates and compared by log-rank analysis. The Mann Whitney-U test was used for the statistical analysis of cytokine data and clinical scores. $P<0.05$ was considered statistically significant.

EXAMPLE 2

Donor pre-treatment with recombinant human G-CSF prevents GVHD in a dose-dependant fashion

The present investigators examined the effect of incrementally increasing the dose of G-CSF administered to SCT donors in a well-established murine SCT model (C57BL/6 Ly5^{a} → B6D2F1) that induces GVHD to major and minor histocompatibility antigens. Although this model utilises spleen as a stem cell source rather than peripheral blood, it's validity has been proven by informative data indicating beneficial effects of G-CSF on both GVHD and GVL (Pan *et al*, 1995; Pan *et al*, 1999) that have since been confirmed clinically (Bensinger *et al*, 2001). Allogeneic donor C57BL/6 animals received 6 daily injections of either control diluent, 0.2μg human G-CSF, 2μg human G-CSF or 10μg human G-CSF and spleens were harvested on day 7. B6D2F1 recipient mice received 1100 cGy of TBI, and splenocytes (corrected to administer 3 x10^{6} T cells per inoculum) transplanted intravenously from respective donors the following day. As shown in FIG. 1a, GVHD induced in this model is severe with all recipients of control splenocytes dying within two weeks with characteristic features of GVHD (weight loss, hunching, fur ruffling, etc). In contrast, 100% of non-GVHD controls transplanted with syngeneic splenocytes survived, confirming that this splenocyte dose contained sufficient stem cells to rescue lethally irradiated recipients. Donor pre-treatment with 0.2μg, 2.0μg or 10.0μg of human G-CSF per day for six days resulted in dose-dependant protection from GVHD lethality, with allogeneic SCT recipient survival at day +60 of 0%, 11% or 50% respectively (P<0.05). Clinical GVHD, assessed by clinical in surviving animals, demonstrated that G-CSF did not completely prevent

GVHD, but donor pre-treatment with G-CSF 10μg/day provides greater protection than mobilisation with 2μg/day or 0.2μg/day (*P*<0.05).

EXAMPLE 3

Donor pre-treatment with murine G-CSF provides equivalent protection to human G-CSF from GVHD at a 10-fold lower dose

The present investigators sought to determine the relative efficacy of murine G-CSF to prevent GVHD compared to human G-CSF. Allogeneic donor C57BL/6 animals received 6 daily injections of either control diluent, 0.2μg murine G-CSF, 0.5μg murine G-CSF or 2μg murine G-CSF. As shown in FIG. 1b, donor pre-treatment with 0.2μg, 0.5μg or 2μg of murine G-CSF again provided dose dependant protection from GVHD lethality, with survival at day 60 of 17%, 33% or 75% respectively (*P*<0.05). Survival at day 60 for recipients of splenocytes pre-treated with 0.2μg of murine G-CSF was equivalent to recipients of splenocytes pre-treated with a ten-fold higher dose of human G-CSF (0.2μg murine G-CSF day 60 survival 17% versus 2.0 μg human G-CSF day 60 survival 11%, *P*=0.63).

EXAMPLE 4

Donor pre-treatment with peg-G-CSF is markedly superior to standard G-CSF in preventing graft-versus-host disease

The present investigators next examined whether the increase in plasma half-life attributable to pegylation of G-CSF led to increased protection from GVHD. Allogeneic donor C57BL/6 animals received either

control diluent, 2μg/day for 6 days of standard G-CSF, or a single dose of peg-G-CSF (3 or 12μg) at day -6. Lethally irradiated B6D2F1 recipient mice were transplanted as above, and grafts were normalised to contain equal numbers of T cells. As shown in FIG. 2a, donor pre-treatment with 3μg or 12μg peg-G-CSF resulted in 83% recipient survival at day 60. Donor pre-treatment with 12μg peg-G-CSF provides significantly more protection from GVHD lethality than the same dose of "standard" human G-CSF given over 6 days ($P<0.0001$). GVHD clinical scores (weight loss, hunching, fur ruffling, etc) were significantly lower in recipients of peg-G-CSF pre-treated spleen compared with recipients of G-CSF treated splenocytes ($P<0.05$ at time points as shown FIG. 2b). In addition, histological examination was perform on liver, skin and bowel of surviving animals receiving grafts from donors pre-treated with peg-G-CSF (data not shown).

EXAMPLE 5

<u>Cellular expansion following donor pre-treatment with standard and pegylated G-CSF</u>

G-CSF has been shown to alter APC phenotype in stem cell grafts, and the present investigators have shown that this contributes to the attenuation of GVHD. We therefore examined both overall spleen expansion and cellular composition following G-CSF or peg-G-CSF pre-treatment. Donor pre-treatment with 2μg per day of standard G-CSF for 6 days lead to an average 53% increase in spleen size (control versus 2μg/day G-CSF for 6 days P<0.0001). Pre-treatment with a single dose of 12μg peg-G-CSF lead to an average 65% increase in spleen size (control versus 12μg peg-G-CSF

day –7 $P<0.0001$). The difference in spleen size between 2μg G-CSF for 6 days and 12μg peg-G-CSF as a single dose was not statistically significant ($P=0.11$).

Pre-treatment with 12μg peg-G-CSF did not alter the total T cell number or sub-set proportions, and in particular the numbers of $CD11c^{+}$ DC and $CD4^{+}CD25^{+}$ regulatory T cells were not altered (FIGS. 3a and 3b). The granulocyte lineage was expanded twofold in peg-G-CSF treated spleens and bone marrow, and to a lesser degree in G-CSF treated spleens (data not shown). As shown in FIGS. 3a and 3b, a novel population of GM cells, defined by a $CD11b^{pos}/Gr\text{-}1^{dim}$ phenotype, were disproportionately increased relative to other APC subsets in peg-G-CSF treated donors (G-CSF versus peg-G-CSF $P=0.001$).

EXAMPLE 6

Donor treatment with peg-G-CSF impairs T cell function and induces regulatory T cell activity

GVHD induced in these models is dependant on T cell function, (Pan et al, 1999; Teshima et al, 1999) and we therefore examined the effect of G-CSF and peg-G-CSF on T cell function in vitro. C57BL/6 T cells were stimulated with alloantigen and T cell proliferation and cytokine production was determined. Pre-treatment of donors with both G-CSF and peg-G-CSF inhibited T cell proliferation to alloantigen, but did not prevent IL-2 production (FIG. 4a). Interferon-γ secretion to alloantigen was reduced 10-fold following donor treatment with peg-G-CSF. Donor T cells from peg-G-CSF animals in response to mitogen (CD3 and CD28) were also reduced 10-fold both pre

and post transplant relative to T cells from control treated donors (data not shown). Since the impairment of T cell proliferation was not associated with reductions in IL-2 production, the investigators next sought to determine whether T cells from cytokine pre-treated donors exhibited regulatory function and were able to inhibit the proliferation of T cells from control treated donors. T cells from non-cytokine exposed C57BL/6 donors were stimulated with alloantigen, with or without the addition of T cells from wild-type of IL-$10^{-/-}$ donors, pre-treated with a single dose (12μg) of peg-G-CSF. As shown in FIG. 4b, T cells from peg-G-CSF pre-treated wild-type donors markedly reduced proliferation ($P<0.05$ at all T cell doses as shown). T cells from IL-$10^{-/-}$ donors impaired proliferation, but to a lesser degree (FIG. 4b) suggesting that IL-10 production is required by donor T cells, at least in part, to provide a regulatory function. Since IL-10 appeared to be playing a role in the inhibition of T cell function from peg-G-CSF treated donors *in vitro*, the investigators next studied an ability of grafts from these animals to produce IL-10 in response to inflammatory stimuli. Surprisingly, spleen from both G-CSF and peg-GCSF treated donors produced 8-fold more IL-10 in response to LPS and CPG relative to control treated spleen (FIG. 4c).

EXAMPLE 7

<u>The protection from GVHD is dependent on production of IL-10 from the donor T cell</u>

Splenocytes pre-treated with peg-G-CSF produced large amounts of IL-10 in response to inflammatory stimuli and T cells from peg-G-

CSF pre-treated donors regulated proliferation of allo-antigen stimulated T cells in vitro in an IL-10 dependant fashion. The investigators therefore next examined whether the protection from GVHD afforded by peg-G-CSF was dependant on IL-10 production by the donor T cell, the non-T cell compartment, or both. C57BL/6 donors in which the IL-10 gene has been homologously deleted (IL-$10^{-/-}$) were pre-treated with 12μg peg-G-CSF on day -6. Wild type T cell depleted (TCD) splenocytes from non-cytokine pre-treated donors plus purified T cells from either wild-type or IL-$10^{-/-}$ donors were infused into lethally irradiated B6D2F1 recipients (FIGS. 12a and 12b). Survival at day 60 was 100% in recipients of wild-type TCD and IL-$10^{-/-}$ TCD spleen alone, confirming that adequate numbers of stem cells were transferred to allow haemopoietic reconstitution. Recipients of allogeneic wild-type T cells had delayed mortality (FIG. 5a) and moderate GVHD as assessed by clinical scores (FIG. 5b), regardless of whether the non-T cell component was from wild-type or IL-$10^{-/-}$ donors. In contrast, recipients of allogeneic IL$10^{-/-}$ T cells all died from GVHD by day 30 regardless of whether the non-T cell component was from wild-type of IL-$10^{-/-}$ donors. Thus, the production of IL-10 by donor T cells is causally associated with protection from GVHD afforded by donors pre-treated with peg-G-CSF. In contrast, IL-10 production by the non-T cell compartment did not influence GVHD.

EXAMPLE 8

The IL-10 producing protective donor T cell has regulatory function

Since the protection from GVHD afforded by peg-G-CSF administration was dependent on IL-10 production by the donor T cell, the present investigators next studied whether these T cells were able to induce infectious tolerance. T cells from wild-type donors pre-treated with control diluent or peg-G-CSF, or IL-$10^{-/-}$ donors pre-treated with peg-G-CSF, were added to wild-type T cell replete grafts from untreated donors. As shown in FIG. 6, the addition of T cells from control treated donors to control grafts did not prevent GVHD mortality with all animals dying by day 12. In contrast, the addition of T cells from peg-G-CSF treated donors to control grafts resulted in 45% survival at day 50 ($P<0.001$). This ability to regulate GVHD was significantly greater in T cells from peg-G-CSF treated donors compared to donor T cells from standard G-CSF treated donors since the later provided only a modest 10 day delay in mortality. The regulation of GVHD by T cells from peg-GSF treated donors was largely, although not completely, dependant on IL-10 production by the donor T cell, since T cells from peg-G-CSF pre-treated IL-$10^{-/-}$ donors delayed, but did not prevent GVHD mortality.

EXAMPLE 9

Discussion

The present investigators show that donor pre-treatment with recombinant human G-CSF protects recipients from GVHD in a dose dependant fashion. Also, treatment of mice with murine G-CSF is approximately 10-fold more potent than human G-CSF, indicating that G-CSF comprising an amino acid sequence that is the same as or similar to that of the donor species is preferred. In addition, donor pre-treatment with a

single dose of peg-G-CSF significantly reduces GVHD when compared with the same dose of standard G-CSF given over 6 days. The protection from GVHD is dependant on donor T cell production of IL-10, and T cells from cytokine pre-treated donors have transferable regulatory activity both *in vivo*.

Species-specific G-CSF (i.e. murine G-CSF in murine transplants) was able to confer equivalent GVHD protection at a 10-fold lower dose than human G-CSF in a murine model. This is likely to reflect superior ligand-receptor interaction between murine G-CSF and the murine G-CSF than between human G-CSF and murine G-CSF receptors. Pegylation of G-CSF significantly increases the plasma half-life of G-CSF, without altering receptor affinity. Thus, not being bound by theory, increased receptor occupancy over a prolonged period leads to further increases in therapeutic efficacy, with significantly improved survival of animals receiving splenocytes from donors pre-treated with a single dose of peg-G-CSF, compared with recipients receiving splenocytes from donors pre-treated with the same dose of standard G-CSF over 6 days.

The present investigators demonstrate that peg-G-CSF leads to an approximate 4-fold expansion of a novel GM APC population, which may be involved with the improvement in regulatory T cell function following donor pre-treatment with peg-G-CSF compared to G-CSF.

$CD4^{+}CD25^{+}$ regulatory T cells have been shown to regulate both autoimmune disease (Sakaguchi *et al*, 1995; Salomon *et al*, 2000), the rejection of solid organ transplants (Hara *et al*, 2001) and GVHD (Hoffmann

et al, 2002). Cohen and colleagues (Cohen *et al*, 2002) examined the regulatory effects naturally occurring $CD4^+CD25^+$ T cells (which represent 5-10% of the normal T cell compartment (Levings *et al*, 2001)) in the B6 to B6D2F1 murine SCT model. They reported that removal of the $CD4^+CD25^+$ T cell compartment from a transplant inoculum resulted in earlier GVHD mortality. Addition of $CD4^+CD25^+$ T cells reduced, although did not prevent, GVHD mortality. Due to the low numbers of $CD4^+CD25^+$ T cells in the peripheral blood healthy donors, stimulation with allogeneic APCs and IL-2 was utilised to induce *ex vivo* expansion. The $CD4^+CD25^+$ T cells retained their regulatory properties. A significant limitation of this approach, however, was the limited half-life of transferred regulatory T cells, with the dramatic appearance of severe lethal GVHD after only a few weeks. Treatment with peg-G-CSF does not lead to expansion of $CD4^+CD25^+$ T cells, and the regulatory T cell induced by peg-G-CSF in relation to the present invention provide long-lasting transplant tolerance. Thus the protective IL-10 producing T cell does not appear to be a classical $CD4^+CD25^+$ T cell, but is likely to be $CD4^+$.

Peg-G-CSF is markedly superior to G-CSF for the long-term prevention of GVHD following allogeneic haematopoietic stem cell transplantation due to the generation of IL-10 producing regulatory donor T cells. These data support the initiation of prospective clinical trials examining the ability of peg-G-CSF mobilised allogeneic peripheral blood stem cell grafts to induce transplant tolerance in both stem cell and solid organ

settings. Furthermore, the induction of IL-10 producing regulatory T cells following peg-GCSF administration suggests applicability to a wider variety of diseases characterised by autoimmunity and failure of regulatory tolerance to self antigens.

It is understood that the invention described in detail herein is susceptible to modification and variation, such that embodiments other than those described herein are contemplated which nevertheless falls within the broad scope of the invention.

The disclosure of each patent and scientific document, computer program and algorithm referred to in this specification is incorporated by reference in its entirety.

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DATED this twenty second day of August 2003

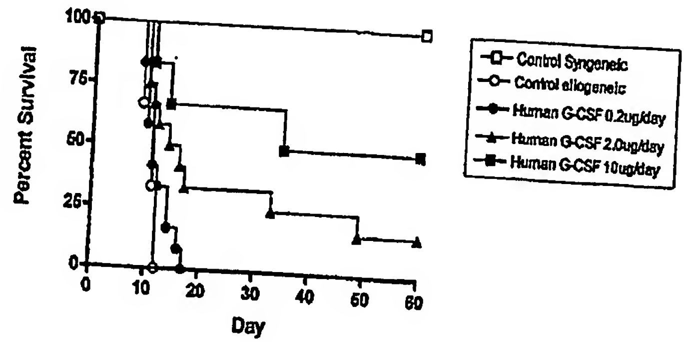
<u>THE COUNCIL OF THE QUEENSLAND INSTITUTE OF MEDICAL RESEARCH</u>

By their Patent Attorneys

Fisher Adams Kelly

Figure 1:

a


Percent Survival
100
75
50
25
0
0
10
20
30
40
50
60
Day
Control Syngeneic
Control allogeneic
Human G-CSF 0.2ug/day
Human G-CSF 2.0ug/day
Human G-CSF 10ug/day

b

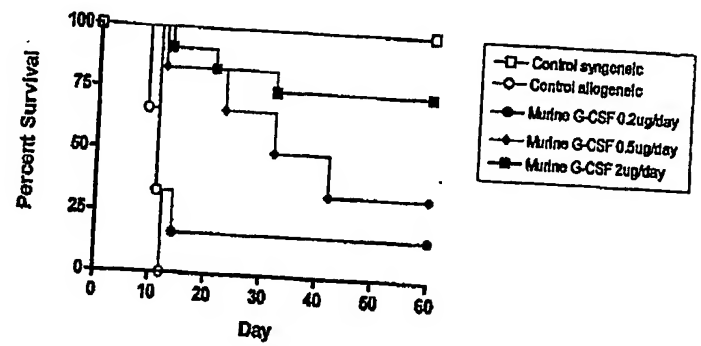
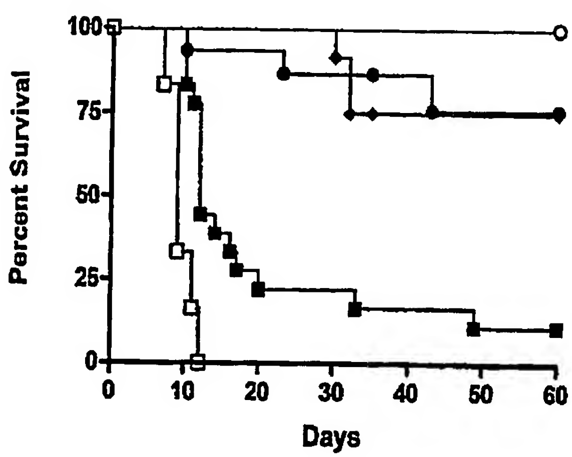

Percent Survival
100
75
50
25
0
0
10
20
30
40
50
60
Day
Control syngeneic
Control allogeneic
Murine G-CSF 0.2ug/day
Murine G-CSF 0.5ug/day
Murine G-CSF 2ug/day

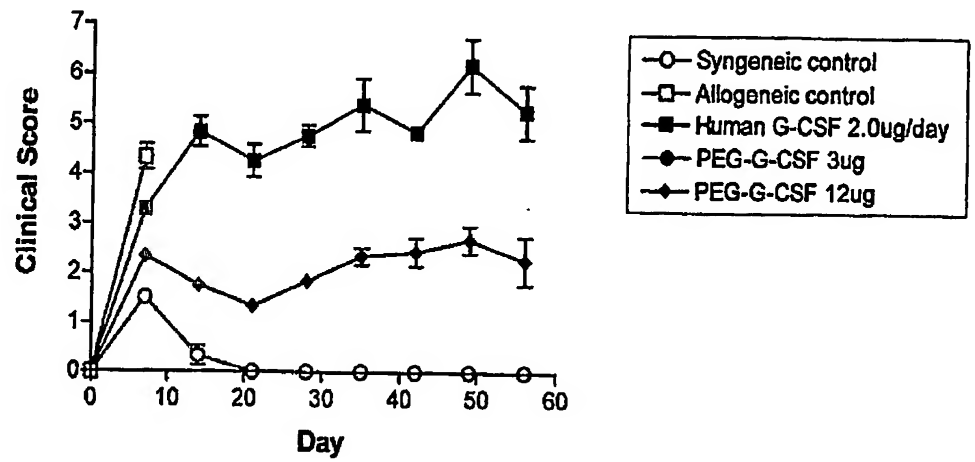
Figure 2:

a



100
75
50
25
0
Percent Survival
0
10
20
30
40
50
60
Days

b



7
6
5
4
3
2
1
0
Clinical Score
0
10
20
30
40
50
60
Day
Syngeneic control
Allogeneic control
Human G-CSF 2.0ug/day
PEG-G-CSF 3ug
PEG-G-CSF 12ug

Figure 3:

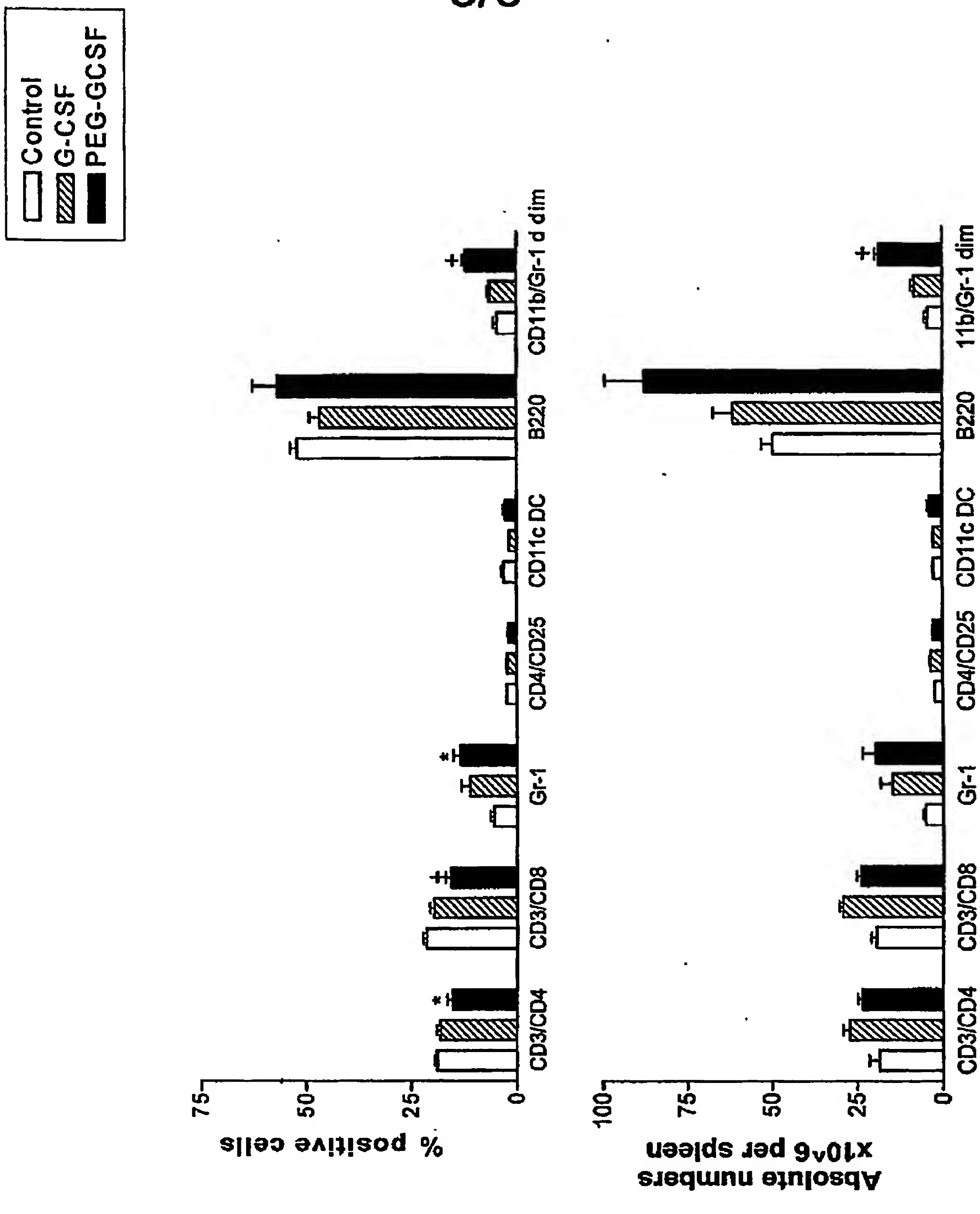
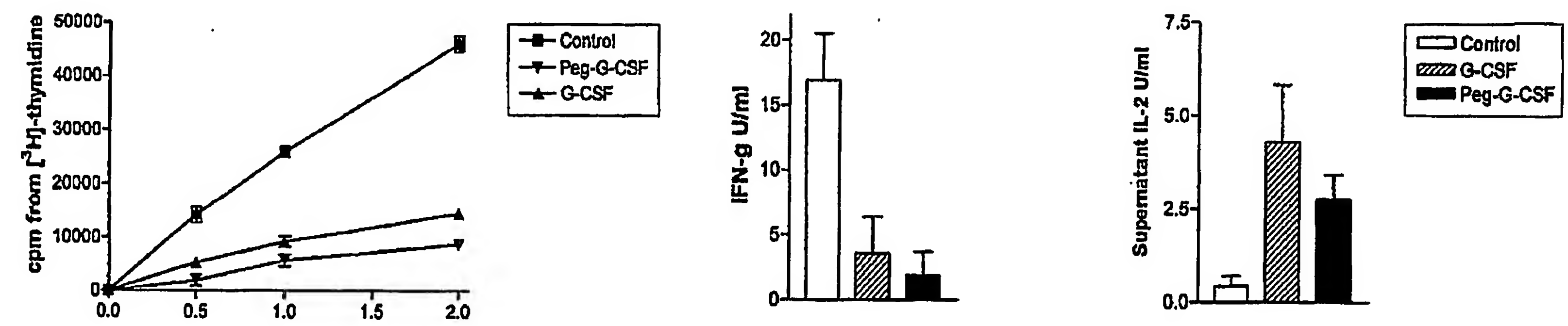

a
b
Control
G-CSF
PEG-GCSF
% positive cells
Absolute numbers x10^6 per spleen
75
50
25
0
100
CD3/CD4
CD3/CD8
Gr-1
CD4/CD25
CD11c DC
B220
CD11b/Gr-1 d dim
11b/Gr-1 dim
*
+

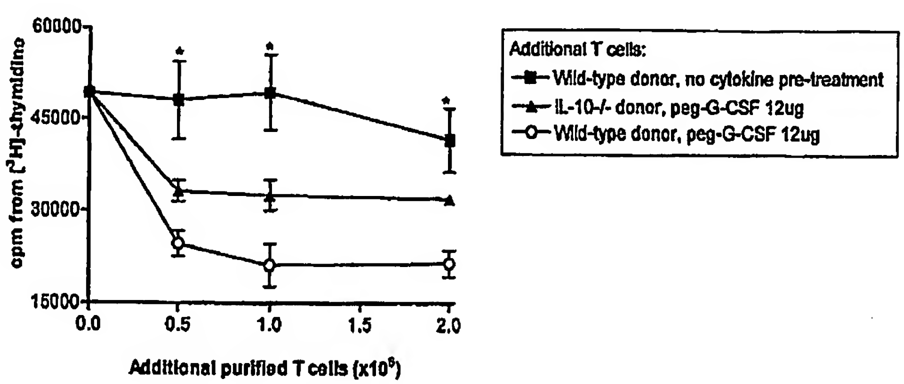
Figure 4:

a



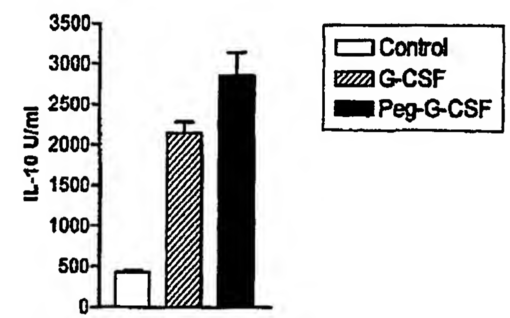
cpm from [3H]-thymidine
50000
40000
30000
20000
10000
0
0.0
0.5
1.0
1.5
2.0
Control
Peg-G-CSF
G-CSF
IFN-g U/ml
20
15
10
5
0
Supernatant IL-2 U/ml
7.5
5.0
2.5
0.0
Control
G-CSF
Peg-G-CSF

b



cpm from [3H]-thymidine
60000
45000
30000
15000
0.0
0.5
1.0
1.5
2.0
Additional purified T cells (x10⁶)
*
Additional T cells:
Wild-type donor, no cytokine pre-treatment
IL-10-/- donor, peg-G-CSF 12ug
Wild-type donor, peg-G-CSF 12ug

c



IL-10 U/ml
3500
3000
2500
2000
1500
1000
500
0
Control
G-CSF
Peg-G-CSF

Figure 5:

a b

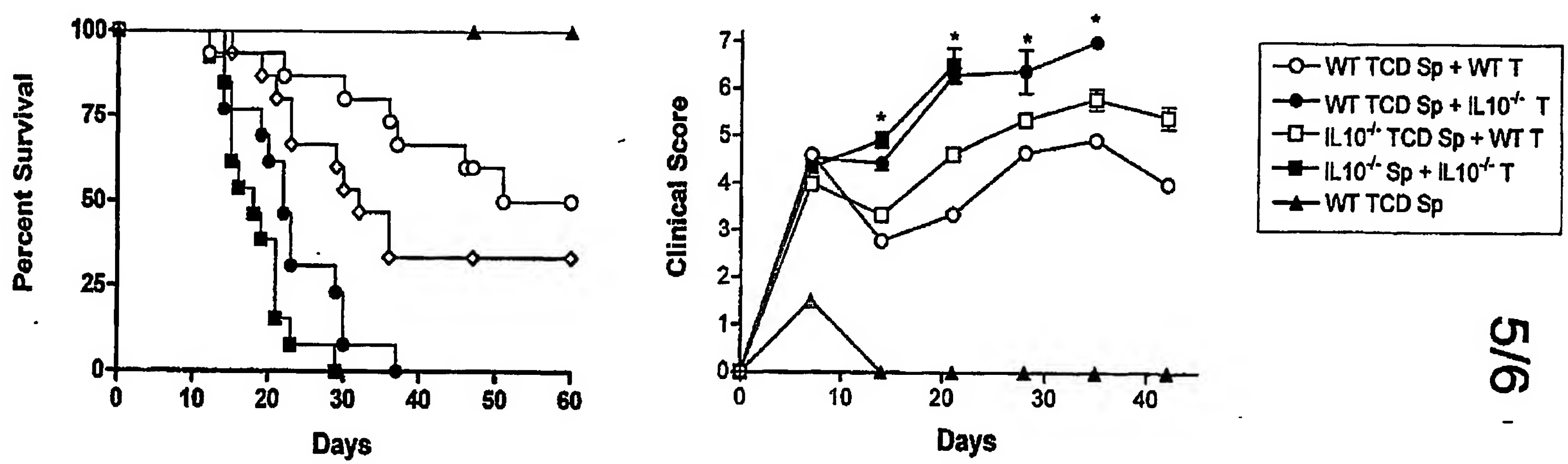
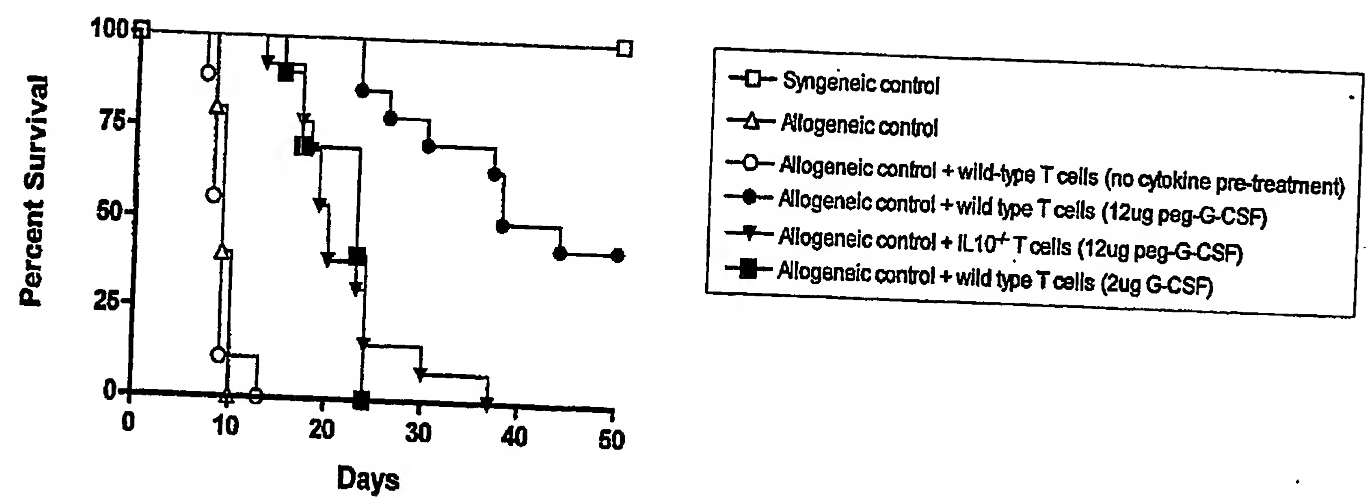

Percent Survival
100
75
50
25
0
0
10
20
30
40
50
60
Days
Clinical Score
7
6
5
4
3
2
1
0
0
10
20
30
40
Days
*
WT TCD Sp + WT T
WT TCD Sp + IL10-/- T
IL10-/- TCD Sp + WT T
IL10-/- Sp + IL10-/- T
WT TCD Sp

Figure 6:


Percent Survival
100
75
50
25
0
0
10
20
30
40
50
Days
Syngeneic control
Allogeneic control
Allogeneic control + wild-type T cells (no cytokine pre-treatment)
Allogeneic control + wild type T cells (12ug peg-G-CSF)
Allogeneic control + IL10-/- T cells (12ug peg-G-CSF)
Allogeneic control + wild type T cells (2ug G-CSF)